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Synergistic activity of polynuclear aromatic hydrocarbon mixtures as aryl hydrocarbon (Ah) receptor agonists†

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Abstract

The relative potencies of benzo[*a*]pyrene and a complex mixture of polynuclear aromatic hydrocarbons (PAHs) produced as by-products of manufactured gas plant (MGP) residues as inducers of hepatic microsomal ethoxyresorufin *O*-deethylase (EROD) activity were determined in the B6C3F1 mouse. The ED_{50} values for the induction response were 78 and 65 mg/kg for benzo[*a*]pyrene and the MGP-PAH mixture, respectively. Analysis of the MGP-PAH mixture indicated that benzo[*a*]pyrene and other compounds containing four or more rings and which are known to induce EROD activity were only present as trace components of this mixture. A comparison of the EROD induction potencies of benzo[*a*]pyrene and the MGP-PAH mixture showed that the mixture was approximately 706 times more potent than expected based on its benzo[*a*]pyrene content (0.17%). This induced P-450 activity could significantly increase the metabolism of the carcinogenic PAHs and thereby modulate the overall carcinogenicity of the mixture. The apparent synergistic activity of the MGP-PAH mixture was further investigated by comparing the activities of this mixture and benzo[*a*]pyrene for several other aryl hydrocarbon (Ah) receptor-mediated responses including (i) induction of hepatic CYP1A1 mRNA levels, (ii) transformation of the rat cytosolic Ah receptor to a complex which binds to a dioxin responsive element, (iii) induction of EROD

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activity and (iv) antiestrogenicity in MCF-7 human breast cancer cells, and (v) inhibition of the splenic plaque-forming cell (PFC) response to both T cell-dependent and independent antigens in B6C3F1 mice. For the EROD and CYP1A1 mRNA induction and cytosolic transformation activities and immunosuppressive effects, the MGP-PAH mixture was approximately 100–900 times more potent as an Ah receptor agonist than expected based on its benzo[*a*]pyrene content. The synergistic activity was lower (19-fold) for the antiestrogenic response in MCF-7 cells. The reason for the synergistic effects of the MGP-PAH mixture were not due to contamination of the mixture by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds and the results suggest that the enhanced potency of the mixture is due to unknown interactions between the individual PAHs present in the mixture.

Key words: Polynuclear aromatic hydrocarbons; Synergistic interactions

1. Introduction

Complex mixtures of polynuclear aromatic hydrocarbons (PAHs) have been widely identified as components of various petroleum products and are formed as by-products from the combustion of organic material [1–7]. The potential human health hazards associated with exposure to PAH mixtures have been reported in several studies and their carcinogenicity to laboratory animals and mutagenic activities have been extensively investigated [4,8–15]. Moreover, results of studies with various PAH mixtures and reconstituted PAH mixtures indicate that a high proportion of their carcinogenicity is due to individual compounds with more than three aromatic rings. This observation was not surprising since most of the carcinogenic PAHs such as benzo[*a*]pyrene, benz[*a*]anthracene, chrysene, dibenz[*a,h*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and the alkylated PAHs such as 7,12-dimethylbenz[*a*]anthracene and 3-methylcholanthrene contain this structural feature [15].

The carcinogenicity of individual PAHs requires metabolic activation and conversion into their corresponding ultimate carcinogenic metabolites which are responsible for DNA alkylation and the initiation step in the complex mechanisms associated with chemically-induced cancer. The metabolic pathways responsible for the formation of genotoxic PAH metabolites have been extensively investigated and reviewed [16–19]. For example, benzo[*a*]pyrene is converted into the highly reactive 7,8-dihydrodiol-9,10-epoxide metabolite via initial P-450-catalyzed formation of the 7,8-oxide followed by hydrolysis to the 7,8-dihydrodiol and a second oxidation step to form the genotoxic diol-epoxide metabolite. The active PAH epoxide metabolites are products of P-450-catalyzed reactions and one particular isozyme, P-4501A1, plays an important role in metabolic activation of several carcinogenic PAHs [16–24]. P-450 isozymes are not only important for the formation of carcinogenic metabolites but also for the generation of other metabolites which are further conjugated and excreted.

The *CYP1A1* gene is constitutively expressed at low to non-detectable levels in most rodent tissues and transformed cell lines but is inducible by several carcinogenic PAHs including benzo[*a*]pyrene [16–24]. Moreover, it has also been reported

that a number of other carcinogenic PAHs including 3-methylcholanthrene, benz[*a*]anthracene, picene chrysene and 7,12-dimethylbenz[*a*]anthracene induce P-4501A1-dependent aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin *O*-deethylase (EROD) activities in rat hepatoma H-4IIE cells in culture [21]. Not surprisingly, all of these inducers also bind with moderate to high affinity to the aryl hydrocarbon (Ah) receptor protein which is known to mediate this induction response [24,25]. The carcinogenicity of municipal gas plant (MGP) PAH mixtures in B6C3F1 mice is currently being investigated in our laboratories. Since the carcinogenicity of PAHs depends, in part, on their activities as inducers of P-4501A1, the present study utilizes an MGP-PAH mixture containing relatively low levels of individual carcinogenic PAHs and investigates the activity of this mixture as an inducer of P-4501A1 and other Ah receptor-dependent responses.

2. Materials and methods

2.1. Chemicals and biochemicals

The crude MGP-PAH mixture used in this study was obtained from the Electric Power Research Institute and chemical analysis of this mixture was determined by META Environ., Inc. GCMS analysis of this mixture identified the following individual PAHs and their percent composition: indan (0.041%), naphthalene (4.3%), 2-methylnaphthalene (4.2%), 1-methylnaphthalene (2.4%), acenaphthalene (1.4%), acenaphthene (0.1%), dibenzofuran (0.12%), fluorene (0.77%), phenanthrene (1.9%), anthracene (0.61%), fluoranthene (0.53%), pyrene (0.78%), benz[*a*]anthracene (0.26%), chrysene (0.27%), benzo[*b*]fluoranthene (0.15%), benzo[*k*]fluoranthene (0.07%), and benzo[*a*]pyrene (0.17%). The other components identified in this mixture include: percent ash after combustion (0%); total organic carbon (90%); cyanide (<0.0001%); inorganic arsenic, berrylium, cadmium, chromium, lead, nickel, selenium and vanadium (<0.006%); alkyl benzenes (<0.04%); and heteroPAHs (<0.025%). The carcinogenicity of this MGP-PAH mixture is currently being investigated and has therefore been utilized in most of the studies reported in this paper. Soxhlet extraction of the crude MGP-PAH mixture with hexane gave a 51% recovery of hexane-soluble fraction and the remainder of this material was a black powdered residue (Alan Tischler, unpublished results). The relative concentration of individual PAHs in this 'cleaned-up' mixture was similar to that reported for the crude mixture but the overall benzo[*a*]pyrene content was increased to 0.55%. Benzo[*a*]pyrene was purchased from the Sigma Chemical Company (St. Louis, MO); the synthetic dioxin responsive element (DRE) (a 26-mer) used in the transformation-gel shift assay was prepared as previously described [26,27]. TCDD, [^{3}H]TCDD (37 Ci/mmol), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and ethoxyresorufin were prepared at Texas A&M University. MCF-7 cells were obtained from the American Type Cell Culture Collection and sheep red blood cells (SRBCs) Alsevars' solution from M.A. Bioproducts, MD. Guinea pig complement, RPMI 1640 media and Earls Balanced Salt Solution were purchased from GIBCO Laboratories, NY. Trinitrophenyl lipopolysaccharide (TNP-LPS Sigma No. T4020), picryl sulfonic acid and glycyl-glycine were purchased from Sigma Chemical Company. NADP, NADPH, and bovine serum albumin (BSA) were purchased from Sigma

Chemical Co. (St. Louis, MO). All other chemicals were of the highest quality available from commercial sources.

2.2. Treatment of animals

Male Long-Evans rats (21 days old, ~100 g) were obtained from Harlan Laboratories (Houston, TX) and housed two per cage with hardwood bedding. Rats were maintained on a diurnal cycle of 12 h of light/dark with free access to food and water. Female C57BL/6 and male C3H mice were obtained from Charles River Laboratories (Boston, MA) and the National Institutes of Health (Bethesda, MD), respectively. They were bred to obtain the B6C3F1 mice in the Texas A&M University Center for Laboratory Animal Research and Resources. The animals were housed as described above. Chemicals dissolved in corn oil (60 μl/mouse) were administered i.p. to 15-day-old male pups (average weight, 7 g). The PAH mixtures appeared to form a solution in corn oil.

2.3. Isolation of hepatic cytosol and microsomes

Rodent hepatic cytosol and microsomes were prepared as described [43]. Animals were sacrificed by cervical dislocation and livers perfused in situ with ice-cold HEGD buffer (25 nM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), pH 7.8). Livers were homogenized in HEGD (3 ml/g tissue) using a Brinkman homogenizer. The homogenate was centrifuged at 10 000 × *g* for 20 min at 2°C and resulting supernatant recentrifuged at 105 000 × *g* for 1 h at 2°C. The cytosol was stored until use in liquid nitrogen, and the microsomal pellet resuspended to a final concentration of 1.0 mg/ml for enzyme assays. Liver cytosolic Ah receptor levels were determined by sucrose density gradient analysis using [^{3}H]TCDD as the radioligand as described [28].

2.4. Cell culture growth

MCF-7 human breast cancer cells were grown in DME/F12 media (Gibco, Grand Island, NY) supplemented with 2.2 g/l tissue grade sodium bicarbonate, 0.2 g/l bovine serum albumin, 10 mg/l transferrin, 5% fetal calf serum (FCS), and a 1% antibiotic-antimycotic solution. Stock cultures were grown in 150 cm^2 culture flasks and incubated at 37°C in 95% air and 5% CO_2. For enzyme assays, 5 × 10^6 cells in 5 ml of media were passaged to 25 cm^2 culture flasks. Solutions of treatment chemicals dissolved in dimethyl sulfoxide (DMSO) were added to the tissue culture flasks so that DMSO final concentration did not exceed 0.1%. Cultured MCF-7 cells were harvested and assayed for enzyme induction 24 h after dosing.

2.5. Induction of EROD activity

EROD activity was determined by the fluorimetric method of Pohl and Fouts [29]. Protein concentrations were determined by the method of Lowry et al. [30].

2.6. Transformation and gel shift assay

A complementary pair of oligonucleotides containing the sequence 5′GATCTGGCTCTTCTCACGCAACTCCG-3′ was synthesized, purified by polyacrylamide gel electrophoresis, annealed, and ^{32}P-labeled at the 5′ end using T4-

polynucleotide kinase and [γ-^{32}P]ATP. DNA binding was measured using a gel shift assay. Rat hepatic cytosol (16 mg/ml protein) was incubated with the test chemicals such that final concentration of DMSO was 0.2% (v/v) for 2 h at 20°C. After incubation, 400 ng of poly [d(I-C)] in HEDG buffer was added and cytosol was incubated for 15 min at 20°C. Following addition of ^{32}P-labeled DRE, the mixture was incubated for an additional 15 min at 20°C. Reaction mixtures were loaded onto a 5% polyacrylamide gel (acrylamide: bisacrylamide, 30:0.8) and electrophoresed at 120 V for approximately 3 h in 0.9 M Tris-borate and 2 mM EDTA, pH 8.0. Gels were dried and protein-DNA interactions determined by imaging on a Betascope 603 blot analyzer imaging system. The amount of ^{32}P-labeled DRE bound in the ligand-inducible complex was estimated by measuring reactivity of the specifically-bound retarded band and subtracting the amount of radioactivity present in the same position in a non-ligand-treated lane [26,27].

2.7. Detection of the secreted 52-kDa protein (procathepsin D)

MCF-7 cells were grown and maintained in RPMI 1640 medium supplemented with FCS which had been treated twice with dextran-coated charcoal (DCC). On day 1, cells were passaged into 150-cm^2 culture flasks containing DME/F12 medium supplemented with 5% FCS treated with DCC (2X) and 3% controlled process serum replacement also treated with DCC (2X). On day 3, cells were seeded into six-well tissue culture plates at a density of 50 000 cells/ml of the DME/F12 medium. On day 4, the medium was changed to the above medium containing 1% FCS treated with DCC (2X). On day 5, cells were washed with PBS and treated with fresh media which contained the appropriate chemicals. After 42 h, the cells were washed with PBS and 750 μl of serum-free DME/F12 medium plus the appropriate chemicals was added. After 6 h, the media was obtained for further analysis. Treatment chemicals were dissolved in absolute ethanol (1% v/v). Secreted proteins were analyzed by SDS-PAGE by loading 5 μg of each sample onto a 15% acrylamide gel slab with a 3% stacking gel. Gels were stained using an 155 ProBlue^{TM} staining kit followed by a standard silver stain procedure, dried, and the 52-kDa protein band was quantified using a laser densitometer as described [31].

2.8. Immunotoxicity studies

The following treatment protocol was used for the immunotoxicity studies. On day 0, mice (4–5 per group) were treated with chemicals in corn oil (10 μl/g body weight). On day 4, animals were immunized with 50 μg of TNP-LPS or SRBCs in 200 μl phosphate buffered saline, and on day 8, mice were sacrificed. A single cell suspension of spleen cells was prepared, washed and resuspended in RPMI 1640 media. The 'Cunningham' modification of the Jerne plaque-forming cell (PFC) assay was used in all experiments [32,33]. Viable spleen cells were determined by trypan blue staining. The number of plaque-forming cells and viable spleen cells were counted with a hemocytometer.

2.9. CYP1A1 mRNA levels

CYP1A1 mRNA levels were measured essentially as described [27] using a 1.2-kb *Pst* I fragment of the murine P1-450 cDNA purchased from ATCC (Rockville, MD).

β-Tubulin mRNA levels were measured using a 1.3-kb *Eco* RI fragment of human β-tubulin cDNA, also purchased from ATCC. Total RNA was isolated by guanidinium thiocyanate/acid phenol extraction. Fifteen μg of total RNA was separated on a 1.2% agarose/1 M formaldehyde gel in 20 mM sodium phosphate and 2 mM CDTA, transferred onto nylon membrane by capillary action, and bound to the membrane by UV crosslinking. The cDNAs were labeled with α[^{32}P]dCTP using a Random Primers DNA Labeling System (BRL) and added at 1–5 × 10^6 counts/min/ml hybridization solution (5X SSPE, 1% SDS, 10% dextran sulfate, 0.1% polyvinyl pyrolidine, 0.1% ficoll, 0.1% bovine serum albumin). Hybridizations were performed in roller bottles at 65°C. The relative intensities of the CYP1A1 and β-tubulin mRNA bands were quantitated on a Betagen Betascope 603 blot analyzer imaging system and subsequently exposed to Kodak X-omat film. The data are expressed as the ratio of CYP1A1 message/βtubulin message ± S.D. for at least four determinations per treatment group.

2.10. Statistics

All treatments were performed at least in triplicate and results presented as means ± S.D. The significant differences between different treatment groups were

Table 1
Dose-dependent induction of hepatic microsomal EROD activity in B6C3F1 mice by MGP-PAH mixtures[a]

Inducer	Dose (mg/kg)	EROD Activity (pmol/mg/min)		CYP1A1 mRNA levels	
		Crude MGP-PAH	Hexane extract	Crude MGP-PAH	Hexane extract
PAH mixtures	0	96 ± 19	150 ± 22	0.14 ± 0.1	1.5 ± 0.5
	33	656 ± 61[c]	1867 ± 87[c]	0.60 ± 0.1[c]	4.5 ± 2.1[c]
	66	1054 ± 176[c]	2234 ± 143[c]	0.80 ± 0.15[c]	6.3 ± 1.4[c]
	132	1332 ± 115[c]	2701 ± 419[c]	1.3 ± 0.3[c]	14.1 ± 7.0[c]
	264	1542 ± 63[c]	3376 ± 53[c]	1.8 ± 0.1[c]	31.4 ± 0.6[c]
	528	1732 ± 116[c]	3704 ± 90[c]	2.1 ± 1.2[c]	33.0 ± 9.0[c]
	1056	2003 ± 225[c]	—	4.2 ± 2.8[c]	—
	2112	1720 ± 156[c]	—	3.4 ± 1.2[c]	—
Benzo[a]pyrene	0	non-detectable		0.56 ± 0.05	
	5	66 ± 15		0.70 ± 0.20	
	15	267 ± 31[c]		1.10 ± 0.45[c]	
	30	583 ± 28[c]		1.0 ± 0.36[c]	
	75	1352 ± 85[c]		2.2 ± 0.57[c]	
	150	2634 ± 310[c]		2.7 ± 0.3[c]	

[a]The B6C3F1 mice (4–5 per group) were treated with the MGP-PAH mixture or benzo[a]pyrene in corn oil; animals were sacrificed after 24 h and hepatic liver microsomes and RNA isolated as described in the Materials and methods section. The results are expressed as means ± S.D.

[b]The hexane extract was obtained after soxhlet extraction of the MGP-PAH mixture for 24 h. The details on this extraction procedure and the recovery of individal PAHs will be reported separately by Dr. A. Tischler.

[c]Significantly different ($P < 0.01$) than the corn oil (vehicle)-treated mice.

determined by ANOVA and Scheffe's or Dunnett's test. EC_{50} or ED_{50} values were determined by probit transformation of the dose-response data [34].

3. Results

The results presented in Table 1 summarize dose-dependent induction of hepatic microsomal EROD activity in B6C3F1 mice by the crude PAH mixture and the hexane extract of this mixture and benzo[*a*]pyrene. Both mixtures caused a dose-response induction of hepatic microsomal EROD activity with significant induction observed at a dose of 33 mg/kg and maximal induction of EROD activity noted at doses of 528 and 1056 mg/kg for the cleaned-up and crude mixtures, respectively; the ED_{50} values for induced EROD activity were 27 and 65 mg/kg, respectively. The results in Table 1 also summarize the dose-response induction of hepatic microsomal EROD activity by benzo[*a*]pyrene. Significant induction was observed at a dose of 30 mg/kg and the ED_{50} value for the induction response was 78 mg/kg. In addition, the dose-response effects of the MGP-PAH mixture, the hexane extract of the mixture, and benzo[*a*]pyrene as inducers of CYP1A1 mRNA levels were determined by northern analysis. The results showed that significant induction for the MGP-PAH mixture, the hexane extract and benzo[*a*]pyrene was observed at doses as low as 33,33 and 15 mg/kg and the ED_{50} values for the induction response were 160, 80 and 27 mg/kg, respectively. The results in Table 2 illustrate the time-dependent induction of hepatic microsomal EROD activity in B6C3F1 mice by the MGP-PAH mixture. Maximal induction was observed after 24 h and significantly-induced EROD activity was determined for up to 7 days after initial treatment. No significant induction was noted 10 or 14 days after treatment with a single dose of this mixture. These data confirm the unexpectedly high activity of the mixture as an inducer of EROD activity and the Ah receptor agonist activities of the MGP-PAHs were further characterized in B6C3F1 mice, rat hepatic cytosol and MCF-7 human breast cancer cells.

Fig. 1. illustrates the sucrose density gradient analysis of the B6C3F1 mouse cytosolic Ah receptor using [^{3}H]TCDD as the radioligand. The specifically-bound

Table 2
Time-dependent induction of hepatic microsomal EROD activity in B6C3F1 mice by the MGP-PAH mixture[a]

Inducer (dose)	Time (days)	EROD Activity (pmol/mg/mg)
MGP-PAH mixture (1056 mg/kg)	1	2739 ± 136[b]
	4	1780 ± 17[b]
	7	543 ± 91[b]
	10	276 ± 19[b]
	14	157 ± 20

[a]The experimental protocol was similar to that described in Table 1 except the animals were sacrificed at different times.
[b]Significantly different ($P < 0.01$) than the corn oil-treated mice.

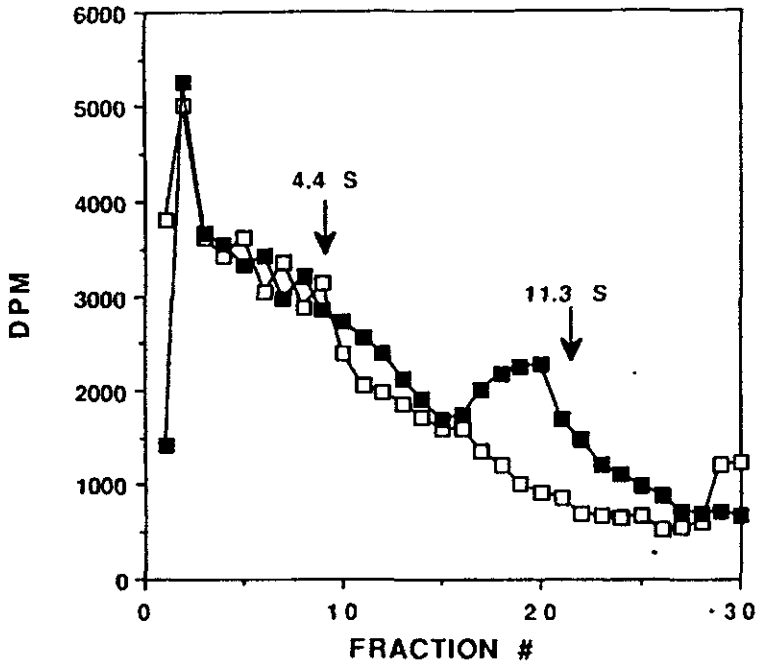

Fig. 1. Sucrose density gradient analysis of the B6C3F1 mouse liver cytosolic receptor. Rat hepatic cytosol was incubated with [^{3}H]TCDD (■) or [^{3}H]TCDD plus a 200-fold excess of unlabeled 2,3,7,8-TCDF (□) to give the levels of total and non-specific binding, respectively. The difference between these values gave the levels of specific binding which sedimented at 10.2 S. The concentration of the cytosolic Ah receptor in B6C3F1 mouse liver was 94 ± 12.7 fmol/mg protein (from three determinations).

receptor complex sedimented at 10.2 S and estimated levels of the Ah receptor were 94 ± 12.7 fmol/mg protein. Results presented in Figs. 2 and 3 summarize the dose-dependent transformation of the rat hepatic cytosolic Ah receptor by the MGP-PAH mixture and benzo[*a*]pyrene. Dose-response curves for the MGP-PAH mixture and benzo[*a*]pyrene were almost superimposable and approximately 100 μg/l caused maximal receptor transformation using a gel mobility shift assay system and a consensus ^{32}P-labeled DRE. The EC_{50} values for transformation of the rat cytosolic Ah receptor by benzo[*a*]pyrene and the MGP-PAH mixture were 2.97 ± 0.60 and 1.78 ± 0.73 μg/l, respectively.

The dose-dependent inhibition of the splenic PFC response to the TNP-LPS antigen was also determined for benzo[*a*]pyrene, the MGP-PAH mixture, and TCDD in B6C3F1 mice. The results (Table 3) show that TCDD caused a significant immunosuppressive response at a dose of 3.6 μg/kg and significantly induced hepatic microsomal EROD activity at a dose of 0.6 μg/kg. The lowest dose of benzo[*a*]pyrene which significantly suppressed splenic PFC response was 100 mg/kg; however, hepatic microsomal EROD activity was not induced at a benzo[*a*]pyrene dose as high as 250 mg/kg. The MGP-PAH mixture exhibited significant immunosuppressive activity at doses of 33–66 mg/kg and hepatic microsomal EROD

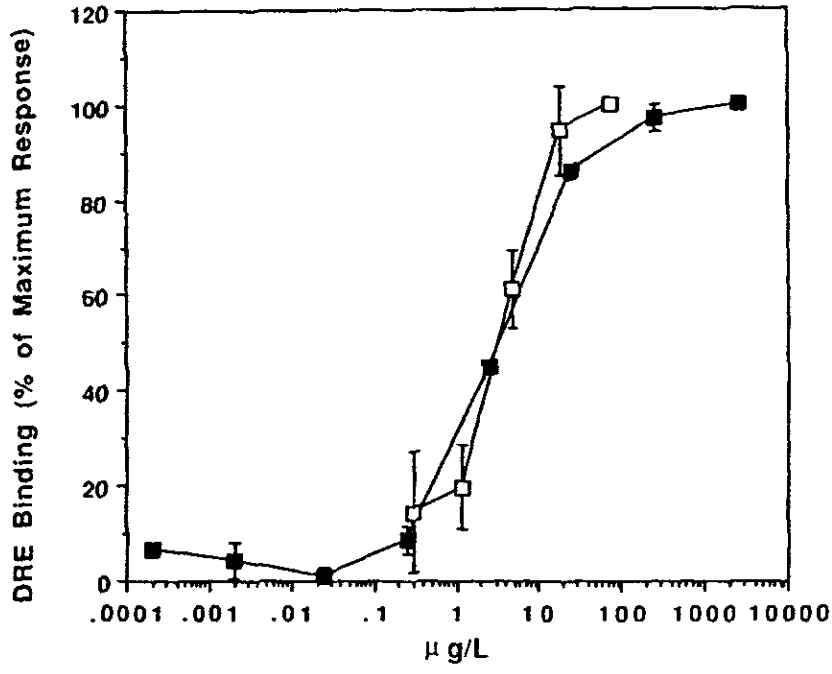

Fig. 2. DRE binding by benzo[*a*]pyrene and the MGP-PAH mixture. The concentration-dependent transformation of rat hepatic cytosol at 20°C by benzo[*a*]pyrene (■) and the MGP-PAH mixture (□) was determined by a gel mobility shift assay [41,42] and the data are expressed as a percentage of the maximal DRE binding observed. The amount of [^{32}P]DRE-transformed Ah receptor complex was quantitated by scanning with a Betagen Betascope 603 blot analyzer imaging system. Incubation of benzo[*a*]pyrene or the MGP-PAH mixture with [^{32}P]labeled DRE and a 50-fold excess of unlabeled DRE resulted in loss of radioactivity in the Ah receptor-DRE complex band (see Fig. 3) (data not shown)

activity was also increased in animals treated with 264 mg/kg of this mixture. The effects of benzo[*a*]pyrene and the MGP-PAH mixture on splenic PFC response to T cell dependent antigen, SRBCs, were also determined in B6C3F1 mice (Table 4). Benzo[*a*]pyrene and the MGP-PAH mixture suppressed splenic PFC response at doses of 10 and 16.5 mg/kg, respectively; however, induced hepatic microsomal EROD activity was only observed for the MGP-PAH mixture at the highest dose and benzo[*a*]pyrene was inactive as an inducer of EROD activity.

The effects of the MGP-PAH mixture as an Ah receptor agonist were also determined in MCF-7 human breast cancer cells. The results presented in Table 5 summarize the concentration-dependent induction of EROD activity in MCF-7 cells by the MGP-PAH mixture. At concentrations of 3900 and 970 μg/l, there was a dose-dependent induction of EROD activity and, at the highest concentration, induced activity decreased slightly due to cytotoxicity. In contrast, 1 μM benzo[*a*]pyrene (252 μg/l) caused only minimal induction of EROD activity in this cell line. The inhibition of 17β-estradiol-induced secretion of the 52-kDa protein by the MGP-PAH mixture

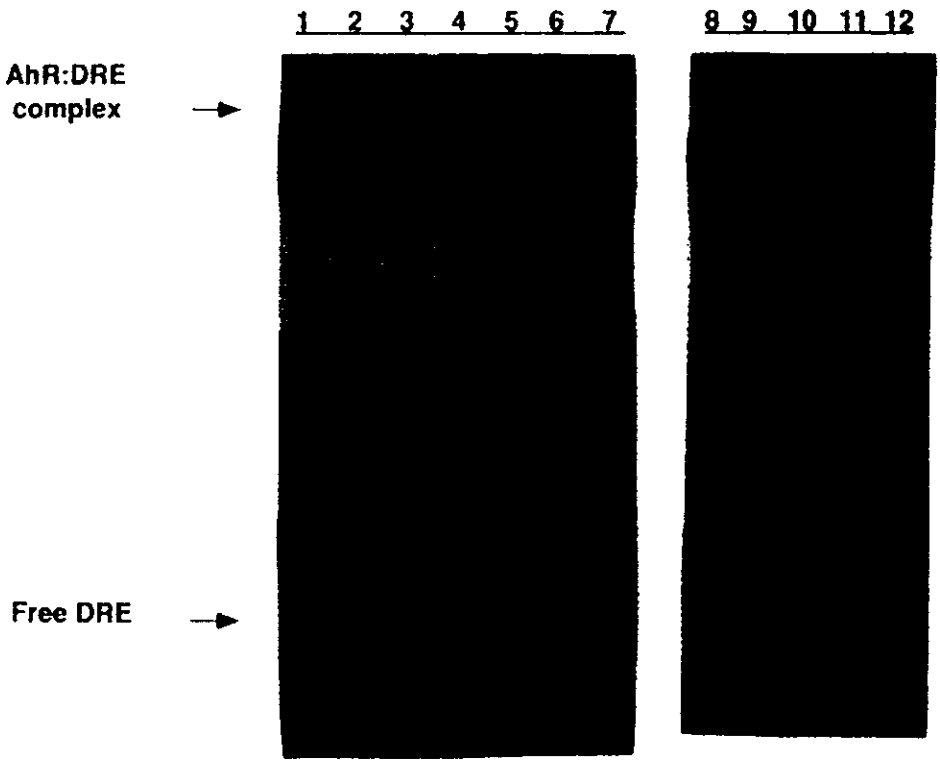

Fig. 3. Gel retardation of rat hepatic cytosol with a ^{32}P-labeled consensus DRE and different concentrations of benzo[*a*]pyrene and the MGP-PAH mixture. The incubation conditions and the separation of the retarded band containing the DRE-Ah receptor complex are outlined in the Materials and methods section. Different concentrations of ligand were used in the transformation assay and these include DMSO (10 μl/ml, lane 1), TCDD (20 μM, lane 2), MGP-PAH mixture (0.3, 1.2, 4.8, 19.1 and 76.3 μg/l, lanes 3–7), benzo[*a*]pyrene (0.252, 2.52, 25.2, 252 and 2,520 μg/l, lanes 8–12). The experiments were determined in triplicate and the results for benzo[*a*]pyrene and the MGP-PAH mixture are summarized in Fig. 2. Incubation of benzo[*a*]pyrene or the MGP-PAH mixture with (^{32}P]labeled DRE and a 56-fold excess of unlabeled DRE resulted in loss of radioactivity in the Ah receptor-DRE complex band.

and benzo[*a*]pyrene was also determined in MCF-7 cells (Table 6). Both the MGP-PAH mixture and benzo[*a*]pyrene caused a concentration-dependent decrease in the 17β-estradiol-induced secretion of the 52-kDa protein and their ED_{50} values were 124 and 4.0 μg/l, respectively.

4. Discussion

Many of the carcinogenic PAHs bind the Ah receptor and exhibit a broad spectrum of Ah receptor-mediated responses including induction of *CYP1A1* gene expression [19–24], induction of other drug-metabolizing enzymes [35,36], decrease in glucocorticoid-receptor binding activity [37], downregulation of epidermal

Table 3
Dose response effects of TCDD, benzo[a]pyrene and the MGP-PAH mixture on the splenic PFC response in B6C3F1 mice treated with the T cell-independent antigen, TNP-LPS[a]

Treatment	Dose (mg/kg)	Spleen cellularity ($\times 10^8 \pm$ S.D.)	PFCs/spleen ($\times 10^5 \pm$ S.D.)	PFCs/10^6 viable cells ($\pm$ S.D.)	EROD activity (pmol/mg/min $\pm$ S.D.)
TCDD	0	1.26 ± 0.37	1.90 ± 0.29	1641 ± 651	215 ± 93
	0.0006	1.23 ± 0.27	1.90 ± 0.18	1603 ± 424	5082 ± 529[b]
	0.0012	1.30 ± 0.43	1.61 ± 0.17	1148 ± 383	5958 ± 1497[b]
	0.0036	1.16 ± 0.19	1.10 ± 0.11[b]	974 ± 208[b]	7665 ± 1050[b]
	0.0072	0.95 ± 0.16	0.74 ± 0.07[b]	793 ± 80[b]	8113 ± 781[b]
Benzo[a]pyrene	0	2.42 ± 0.65	2.84 ± 0.39	1260 ± 468	101 ± 13
	10	2.31 ± 0.11	2.71 ± 0.32	1181 ± 177	77 ± 13
	50	1.93 ± 0.50	2.16 ±0.47[b]	1087 ± 87	83 ± 5
	100	1.70 ± 0.22[b]	1.22 ± 0.23[b]	722 ± 135[b]	78 ± 8
	250	1.30 ± 0.10[b]	1.03 ± 0.24[b]	780 ± 209	104 ± 3
MGP-PAH Mixture	0	1.69 ± 0.32	4.31 ± 0.18	2566 ± 273	158 ± 12
	16.5	1.77 ± 0.16	3.73 ± 0.62	2124 ± 456	185 ± 24
	33	2.14 ± 0.12	3.77 ± 0.43	1767 ± 249[b]	187 ± 34
	66	1.97 ± 0.53	1.68 ± 0.24[b]	884 ± 197[b]	215 ± 33
	264	1.43 ± 0.35	1.68 ± 0.09[b]	1227 ± 284[b]	638 ± 122[b]

[a]Mice (4–5 per group) were injected i.p. with different doses of the chemicals in corn oil on day 0. The mice were then immunized with TNP-LPS by i.p. administration of corn oil on day 4 and animals were sacrificed on day 8. Spleen cellularity, number of PFCs and hepatic microsomal EROD activity were determined as described in the Materials and methods section. The results are expressed as means ± S.D.
[b]Significantly different ($P < 0.05$) than the corn oil-treated mice.

Table 4
Dose response effects of benzo[a]pyrene and the MGP-PAH mixture on the splenic PFC response in B6C3F1 mice treated with the T cell-dependent antigen, SRBCs[a]

Treatment	Dose (mg/kg)	Spleen cellularity ($\times 10^8 \pm$ S.D.)	PFCs/spleen ($\times 10^5 \pm$ (S.D.)	PFCs/10^6 viable cells ($\pm$ S.D.)	EROD activity (pmol/min/mg ($\pm$S.D.)
Benzo[a]pyrene	0	1.66 ± 0.29	4.65 ± 0.69	2775 ± 786	243 ± 14
	10	1.55 ± 0.16	2.79 ± 0.72[b]	1792 ± 334[b]	230 ± 41
	50	2.20 ± 0.31[b]	1.70 ± 0.35[b]	786 ± 210[b]	187 ± 14
	100	1.96 ± 0.31	1.27 ± 0.32[b]	655 ± 150[b]	173 ± 23
	200	1.39 ± 0.21	0.96 ± 0.15[b]	700 ± 128[b]	166 ± 61
MGP-PAH mixture	0	1.72 ± 0.41	5.05 ± 1.55	2928 ± 393	170 ± 27
	16.5	1.47 ± 0.18	1.98 ± 0.30[b]	1409 ± 140[b]	151 ± 13
	33	1.41 ± 0.22	1.69 ± 0.16[b]	1168 ± 639[b]	163 ± 11
	66	1.30 ± 0.26	1.43 ± 0.72	1092 ± 366[b]	192 ± 32
	264	1.59 ± 0.46	1.47 ± 0.84[b]	898 ± 333[b]	577 ± 69[b]

[a]The experimental procedures were similar to those described in Table 3 except that the antigen used was SRBCs.
[b]Significantly different ($P < 0.05$) than the corn oil-treated mice.

Table 5
Induction of EROD activity in MCF-7 cells by the MGP-PAH mixture and benzo[a]pyrene[a]

Treatment	PAH Concentration (μg/l)	EROD Activity (pmol/min/mg)
DMSO	0	non-detectable
MGP-PAH mixture	15	non-detectable
	60	non-detectable
	240	21 ± 0.9[b]
	970	79 ± 5.7[b]
	3900	49 ± 3.5[b]
Benzo[a]pyrene	252	6.3 ± 0.6

[a]MCF-7 cells were treated with different concentrations of the MGP-PAH mixture or benzo[a]pyrene in DMSO for 24 h and EROD activity was determined fluorimetrically as described [44]. Lower concentrations of benzo[a]pyrene were inactive as inducers. The results are expressed as means ± S.D. for at least 3 determinations per treatment group.
[b]Significantly different ($P < 0.01$) than DMSO-treated cells.

Table 6
Inhibition of 17β-estradiol-induced secretion of procathepsin D by the MGP-PAH mixture and benzo[a]pyrene in MCF-7 cells[a]

Treatment	PAH Concentration (μg/l)	Relative levels of secreted procathepsin D
17β-Estradiol (1 nM)	0	411 ± 21[b]
DMSO	0	100 ± 12
MGP-PAH mixture +	15	299 ± 42[c]
1 nM 17β-estradiol	60	222 ± 36[c]
	240	131 ± 22[c]
	970	87 ± 23t[b]
	3900	73 ± 22[c]
MGP-PAH mixture	3900	61.3 ± 9
17β-Estradiol (1 nM)	0	316 ± 24[b]
DMSO	0	100 ± 14
Benzo[a]pyrene +	0.25	207 ± 17[c]
1 nM 17β-estradiol	2.5	102 ± 9[c]
	25	122 ± 11[c]
	252	92 ± 12[c]
Benzo[a]pyrene	252	73 ± 11

[a]MCF-7 cells [50 000] were seeded in multiwell culture plates and after attachment were treated with solvent (DMSO) vehicle control and the various chemicals for 42 h; cells were then washed and treated with the same chemical in serum-free media for 6 h. Secreted proteins were separated by SDS-PAGE, double-stained with ISS ProBlue™ and silver stain and finally quantitated by densitometric analysis as described in the Materials and methods section.
[b]Significantly higher ($P < 0.01$) than levels in DMSO-treated cells.
[c]Significantly lowwer ($P < 0.01$) than levels in 17β-estradiol-treated cells.

growth factor receptor binding activity [38], and inhibition of several 17β-estradiol-induced responses in MCF-7 human breast cancer cell lines [39]. PAHs also exhibit immunosuppressive activity and some of these responses may be mediated, in part, through the Ah receptor [40]. One of the major differences between PAHs and halogenated aromatic hydrocarbons (HAHs), such as TCDD, is associated with the relatively high tissue persistence of the chlorinated compounds. Both chemical classes elicit many of the same short-term Ah receptor-mediated responses such as induction of *CYP1A1* gene expression; however, PAH-induced responses are usually short-lived due to metabolism by the induced P-450 isozymes. In contrast, TCDD and related HAHs are resistant to P-450-mediated metabolism and their subsequent persistent long-term occupation of the Ah receptor is related to development of several delayed toxic responses such as the wasting syndrome, thymic atrophy and tumor promotion activity [41] which are not usually observed with PAHs.

The carcinogenic potencies of MGP-PAH mixtures are currently being investigated in this laboratory. Since P-450 isoenzymes play an important role in the subsequent formation of carcinogenic and non-carcinogenic PAH metabolites, it was therefore of interest to characterize activity of these mixtures as inducers of *CYP1A1* gene expression.

The results presented in Tables 1 and 2 show that benzo[*a*]pyrene and the crude MGP-PAH mixture and a hexane extract (soxhlet) of this mixture caused a dose-dependent induction of hepatic microsomal EROD activity in B6C3F1 mice and this is consistent with the presence of the hepatic cytosolic Ah receptor in these mice (Fig. 1). When the crude MGP-PAH mixture was 'cleaned up' by soxhlet extraction with hexane to remove more polar impurities (A. Tischler, unpublished results), the EROD-inducing potencies of the crude (ED_{50} = 65 mg/kg) and 'cleaned-up' PAH mixtures (ED_{50} = 27 mg/kg) were higher than observed for benzo[*a*]pyrene (ED_{50}, 78 mg/kg). Thus, based on the relatively low benzo[*a*]pyrene levels in the crude MGP-PAH mixture (0.17%), the observed EROD induction response was approximately 706 times more potent than expected based on benzo[*a*]pyrene content alone. The induction of CYP1A1 mRNA levels was also determined in the same animals by Northern analysis of hepatic mRNA extracts. The results (Table 1) showed that the MGP-PAH mixture, the hexane extract of this mixture, and benzo[*a*]pyrene caused a dose-response induction of CYP1A1 mRNA levels in B6C3F1 mouse liver and the MGP-PAH mixture was approximately 99 times more potent as an inducer based on a benzo[*a*]pyrene content of 0.17%. The reason for the differences between the synergistic induction of EROD activity and CYP1A1 mRNA levels by the MGP-PAH mixture are unknown. Inspection of PAHs present in the MGP-PAH mixture (see Materials and Methods section) show that only a small percentage of these congeners are Ah receptor agonists based on their induction activity [26], observed [26] or calculated [49] Ah receptor binding affinities. These include benzo[*a*]pyrene (0.17%), benzo[*k*]fluoranthene (0.07%), benzo[*b*]fluoranthene (0.15%), chrysene (0.27%), and benz[*a*]anthracene (0.26%). While the synergistic induction response would be somewhat decreased if induction potencies of the trace levels of the remaining carcinogenic PAHs in the mixture were included in the calculation, the observed synergistic induction response would still be greater than two orders of magnitude.

The synergistic activity of the MGP-PAH mixture was further investigated by

Table 7
A comparison of the relative potencies of benzo[*a*]pyrene and a MGP-PAH mixture as Ah receptor agonists[a]

Response	Benzo[*a*]pyrene ED_{50}	MGP-PAH Mixture ED_{50}[b]	Synergism
Induction of EROD activity in B6C3F1 mice	78 mg/kg	65 mg/kg	706-fold
Induction of hepatic CYP1A1 mRNA levels in B6C3F1 mice	27 mg/kg	160 mg/kg	99-fold
Induction of EROD activity in MCF-7 cells	>240 μg/l	>252 μg/l	>617-fold
Transformation of the rat cytosolic Ah receptor	2.97 μg/l	1.78 μg/l	911-fold
Immunotoxocity in B6C3F1 mice[c]			
TNP-LPS (antigen)	91.7 mg/kg	27.7 mg/kg	195-fold
SRBCs (antigen)	37.8 mg/kg	27.8 mg/kg	801-fold
Antiestrogenicity in MCF-7 cells	4 μg/l	124 μg/l	19-fold

[a]Derived from the data in Tables 1 and 3 through 6 by probit transformation of the results.
[b]The benzo[*a*]pyrene content of the mixture was 0.17%.
[c]Based on the PFCs/10^6 cells.

comparing the potency of this mixture with that of benzo[*a*]pyrene for several other Ah receptor-mediated responses. Previous studies have demonstrated the structure-dependent transformation of the cytosolic Ah receptor by PAHs [43] and the comparative activities of the MGP-PAH mixture and benzo[*a*]pyrene to transform the cytosolic Ah receptor to a DRE-binding complex are summarized in Figs. 2 and 3. The ED_{50} values for the induced transformation by benzo[*a*]pyrene and the MGP-PAH mixture were 2.97 and 1.78 μg/l and this represented a 911-fold higher response by the mixture based on a benzo[*a*]pyrene content of 0.17%. It has also been suggested that the immunotoxicity of PAHs may also be Ah receptor-mediated [40] and therefore the effects of benzo[*a*]pyrene and the MGP-PAH mixture on the splenic PFC response to SRBCs and TNP-LPS was investigated. The dose-response results (Tables 3 and 4) showed that benzo[*a*]pyrene and the MGP-PAH mixture were immunotoxic and a comparison of their ED_{50} values (Table 7) indicated that the mixture was 195–810-fold more active based on a benzo[*a*]pyrene of 0.17%. Research in this laboratory has previously shown that MCF-7 human breast cancer cells express the Ah receptor [44] and the results of several studies support a role for the Ah receptor in mediating both the induction of *CYP1A1* gene expression and the antiestrogenic effects of halogenated aromatic hydrocarbons and PAHs [39,44–47]. Not surprisingly, a comparison of the potencies of benzo[*a*]pyrene and the MGP-PAH mixture as inducers of EROD activity and as inhibitors of estrogen-induced secretion of the 52-kDa protein showed that the mixture was >617- and 19-fold, respectively, more actively based on a benzo[*a*]pyrene content of 0.17% for the mix-

ture. The increased potency of the MGP-PAH mixture as an antiestrogen in MCF-7 cells was considerably lower (19-fold) than observed for the other responses. This was not surprising since previous studies [39] and the results shown in Table 6 indicate that PAHs exhibit relatively high potencies as antiestrogens compared to other Ah receptor-mediated responses. This results in an overall shift of the dose-response curve for PAHs to lower concentrations, thus decreasing the potency differences between individual PAHs and PAH mixtures.

One possible explanation for the synergistic activities of the MGP-PAH mixture might be the presence of trace quantities of potent halogenated aromatic hydrocarbons such as TCDD and related compounds. However, the results presented in Table 2 summarizing the time-course induction of EROD activity by the MGP-PAH mixture show that induced activity decreases rapidly with time. In contrast, if the induction response was due to the presence of TCDD and related halogenated aromatics as contaminants in the MGP-PAH mixture, then the rapid decrease in EROD activity would not have been observed due to the tissue stability and persistence of these compounds. For example, eight days after administration of TCDD, significant induction of hepatic microsomal EROD activity was observed in B6C3F1 mice whereas benzo[*a*]pyrene and the MGP-PAH mixture did not induce this activity even at high doses which elicted immunosuppressive responses (Table 3). This is also illustrated by the results in Table 2 in which EROD activity induced by the MGP-PAH mixture decreased rapidly with time. Moreover, high resolution GCMS analysis of the MGP-PAH mixture indicated that polychlorinated dibenzo-*p*-dioxin and dibenzofuran levels in the crude mixture were non-detectable at the sub-parts per billion level (Brock Chittim, Wellington Laboratories, personal communication).

In summary, the results of this study demonstrate that a PAH mixture derived from manufactured gas plant processes exhibited non-additive (synergistic) activities as an Ah receptor agonist for several in vivo and in vitro responses. The reason for these interactive effects are unknown; however, they are not related to contamination by trace levels of TCDD and related compounds. It is possible that as yet unidentified compounds which are potent inducers of EROD activity are present in these mixtures and current studies are focused on identifying these chemicals or determining the interactions which are responsible for the non-additive effects of MGP-PAH mixtures as Ah receptor agonists.

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